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Relationships between diabetic nephropathy and insulin resistance, inflammation, Trx, Txnip, CysC and serum complement levels

L.-L. XU, W. GAO, Z.-M. CHEN, K.-K. SHAO, Y.-G. WANG, L.-L CUI, N.-Z. GUO

Department of Laboratory Medicine, The First People's Hospital of Yancheng City, Yancheng, China

Linlin Xu and Wen Gao contributed equally to this work

Abstract. – OBJECTIVE: To investigate the relationships between diabetic nephropathy (DN) and insulin resistance, inflammation, thioredoxin (Trx), thioredoxin-interacting protein (Txnip), Cystatin C (CysC) and serum complement levels.

PATIENTS AND METHODS: A total of 119 patients with type 2 diabetes mellitus (T2DM) treated in the Endocrinology Department of our hospital from January 2017 to December 2017 were enrolled as the experiment group, while 30 healthy volunteers were selected as the control group. The expression levels of inflammatory factors, Trx, Txnip, CysC and serum complements in every subject were detected. In addition, the type 2 diabetic nephropathy rat model was established via high-fat diet and injection of low-dose streptozotocin. Blood glucose, insulin resistance indexes and 24h-urinary albumin excretion were measured, and the histomorphological characteristics of the kidney in animals were observed.

RESULTS: In clinical subjects, Trx level was notably lower in the simple DM group, early DN group and clinical DN group in comparison with that in the control group. The levels of Txnip and CysC in the simple DM group, early DN group and clinical DN group were remarkably higher than those in the control group. Moreover, the expression levels of TNF-α and IL-6 in the clinical DN group were significantly elevated compared with those in the simple DM group and early DN group. In addition, C1q expression in the clinical DN group was higher than that in the simple DM group and early DN group. In model rats, HOMA-IR was distinctly higher in the DM group and DN group than that in the control group. The ratio of kidney weight to body weight (KW/BW) was evidently higher in the DN group in comparison with that in the control group and DM group.

CONCLUSIONS: Insulin resistance, inflammatory factors, and levels of Trx, Txnip, CysC and serum complement C1q are related to the progression of DM.

Key Words:
DN, Insulin resistance, Inflammation, Trx, Txnip, CysC, Serum complement.

Introduction

Diabetes mellitus (DM) has become a global public health problem[1]. Diabetic nephropathy (DN) is the most common cause of chronic kidney diseases, accounting for approximately 50% of DM cases in the developed countries[2]. However, the pathogenesis of DN remains unclear. The main causes of DN are advanced glycation end-products (AGE1) produced due to long-term hyperglycemia, activation of protein kinase C, enhanced expression of transforming growth factor-β (TGF-β), and oxidative stress[3,4]. Increasing evidence has indicated that activation of the complement system is associated with the pathogenesis of DN. Glomerular complement C3 deposition is observed in mice with type 1 DM (T1DM) and T2DM[5]. Glomerular deposition of membrane attack complex (MAC) C5b-9 is found in patients with DM[6]. Besides, C3a-mediated inflammatory and pre-fibrotic responses exacerbate renal damage in T2DM rats, while inhibition of complement C5 can attenuate glomerular mesangial proliferation and urinary protein excretion in rats. It is suggested that the activation of complement system is correlated with the pathogenesis of DN.

Complement system consists of three activation pathways (classical, lectin and alternative pathways). The common product, C3 convertase, is able to activate downstream complement response to form MAC[7]. In recent years, it has been revealed that the lectin pathway may be related to the pathogenesis of DN. In the lectin pathway,

Corresponding Author: Naizhou Guo, BM; e-mail: Guonaizhou@sina120.com

mannose-binding lectin (MBL) and fibrillin recognize and bind to mannose, fucose and N-acetyl-glucosamine on the surface of pathogenic microorganisms. Subsequently, MBL-associated serine proteases (MASPs), mainly MASP-2, are activated, which further activate the downstream components of the complement system[8]. Generally speaking, MBL and fibrin do not bind to their own tissues. However, such an interaction between MBL and fibrin will be induced by glycosylation in DM, leading to systemic and local inflammatory responses, and ultimately diabetic complications[9].

Previous studies demonstrated that MBL is deposited in the glomeruli of T1DM mice. The kidney weight, urinary albumin excretion (UAE) rate, and type IV collagen expression are significantly reduced in MBL-knockout mice with T1DM. Lindhardt et al[10] revealed that patients with T2DM have significantly increased serum level of MBL, which can be used to predict the risk of developing DN. MASP-2 is a key molecule in the lectin-pathway activation. However, it is unclear whether MASP-2 is involved in the pathogenesis of DN. Serum MASP-1 and MASP-2 levels are notably higher in T1DM patients in comparison with those in the control group[11]. Complement factors are mainly synthesized in the liver, while they are activated and cascaded in the blood circulation. The relationship between complement components and DN is rarely reported. Therefore, this study aims to evaluate the expressions of complement components in the kidneys of T2DN rats, so as to investigate their roles in DN. Meanwhile, the effects of insulin resistance, inflammation-related factors, thioredoxin (Trx), thioredoxin-interacting protein (Txnip) and Cystatin C (CysC) on DN were observed.

Patients and Methods

Clinical Data

A total of 119 patients with T2DM treated in the Endocrinology Department of our hospital from January 2017 to December 2017 were enrolled as the experiment group, while 30 healthy volunteers were selected as the control group. Inclusion criteria: a) patients were diagnosed as T2DM according to the criteria proposed by WHO, and b) informed consent was obtained. Exclusion criteria: a) patients with T1DM, b) those complicated with hypertension, malignant tumors, hematological diseases or central nervous system diseases, or c) those who took glucocorticoids, immunosuppressants or nephrotoxic drugs recently. The general information of the subjects in each group were shown in Table I. This investigation was approved by the Ethics Committee of The First People's Hospital of Yancheng City. Signed written informed consents were obtained from all participants before the study.

Establishment of Rat Models

A total of 24 male Sprague-Dawley rats aged 5 weeks old and weighing about 150 g were purchased from the Laboratory Animal Center of Anhui Medical University. The rats were fed under standard conditions and had free accesses to food and water. This study was approved by the Animal Ethics Committee of The First People's Hospital of Yancheng City Animal Center.

Before the experiment, 24 rats were habituated for 1 week. Then, they were randomly divided into three groups: Control group (n=8), T2DM group (DM group, n=8) and T2DN group (DN group, n=8). The rats in control group were fed normally, while those in DM group and DN group were administrated with high-fat diet (Research Diets, D12451) for 8 weeks. Subsequently, fasting insulin (FINS) and fasting plasma glucose (FPG) were measured to calculate the homeostasis model assessment of insulin resistance (HOMA-IR=-FINS×FPG/22.5). Next, rats in the DM group and DN group were injected intraperitoneally (ip) with streptozotocin (STZ) at a low dose of 30 mg/kg, while rats in the control group were injected with the same volume of citrate buffer. The glucose levels were tested at 72 h after STZ injection. DM was confirmed in the rats with glucose levels higher than or equal to 16.7 mmol/L for 3 consecutive days[24]. Rats in the DN group continued to feed high-fat diet for 8 weeks.

ELISA

About 5 mL of fasting venous blood was drawn from the subjects and centrifuged at 3000 r/min for 10 min. The upper layer supernatant was collected. Serum levels of Trx, Txnip and CysC were detected *via* enzyme-linked immunosorbent assay (ELISA) using the commercial kits purchased from Beijing Zhongshan Golden Bridge Bio-technology Co., Ltd. (Beijing, China).

Kidney Pathology

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine. Every effort was made to minimize the pain of animals. Then, kid-

neys were removed and weighed. After the kidney tissues were fixed in 4% paraformaldehyde and paraffin-embedded, they were cut into 4 μm sections, followed by hematoxylin-eosin (HE) and periodic acid-Schiff staining.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20 (IBM, Armonk, NY, USA) was utilized for statistical analysis. Data were expressed by mean ± standard deviation ($\bar{x}\pm s$). Comparison between multiple groups was done using One-way ANOVA test, followed by Post-Hoc Test (Least Significant Difference). Kruskal-Wallis H-test was adopted to analyze the distribution, and Pearson's chi-squared test was conducted for related variables of normal distribution data. Furthermore, Spearman test was applied for analysis of non-normal distribution data. $p<0.05$ suggested that the difference was statistically significant.

Results

Comparisons of Trx, Txnip, and CysC Levels in Subjects

The level of Trx was notably lower in the simple DM group, early DN group and clinical DN group in comparison with that in the control group ($p<0.05$), which was the lowest in the clinical DN group. The levels of Txnip and CysC in the simple DM group, early DN group and clinical DN group were remarkably higher than those in the control group ($p<0.05$), which were the highest in the clinical DN group (Table II).

Levels of Inflammatory Factors in Subjects

The tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels in recruited subjects were determined. Expression levels of TNF-α and IL-6 in the clinical DN group were significantly elevated compared with those in the simple DM group and early DN group ($p<0.05$; Figure 1).

Table I. General information of subjects.

Group	Case	Male/ Female	Age
Simple DM group	32	17/15	53.21±8.15
Early DN group	42	20/22	54.29±7.26
Clinical DN group	45	24/21	53.19±8.21
Control group	30	16/14	52.04±10.24
p		0.632	0.234

Serum Complement Level in Subjects

Serum level of complement C1q in each group of subjects was tested. C1q level in the clinical DN group was higher than that in the simple DM group and early DN group ($p<0.05$; Table III). However, there was no apparent difference in C1q level between control group and simple DM group.

Establishment of T2DN Rat Model

H&E staining was conducted to evaluate glomerular volume, glomerular capillaries, glomerular mesangial area, renal tubular epithelial cells and renal interstitium. Compared with control group and DM group, the main pathological changes in rats of the DN group included glomerular hypertrophy, glomerular mesangial cell proliferation, capillary lobularization and Bowman's capsule narrowing. In addition, the interlobular arterial wall and renal tubule basement membrane showed thickening. Swollen epithelial cells and protein-like casts were observed. However, there were no significant differences in the morphological characteristics of kidney tissues between DM group and control group (Figure 2).

After the high-fat diet for 8 weeks, HOMA-IR was distinctly higher in the DM group and DN

Table II. Comparisons of Trx, Txnip, and CysC levels among groups.

Group	Case	Trx (ng/L)	Txnip (pg/ml)	CysC (mg/L)
SimpleDM group	32	102.02±37.30*	137.81±15.02*	1.20±0.47*
EarlyDN group	42	80.27±31.27$^{*\#}$	142.90±15.48$^{*\#}$	1.42±0.41$^{*\#}$
ClinicalDN group	45	56.04±18.03$^{*\#\&}$	143.50±15.12$^{*\#\&}$	2.13±0.48$^{*\#\&}$
Control group	30	129.02±41.88	113.31±12.21	0.88±0.18
t		199.211	189.412	14.312
p		<0.05	<0.05	<0.05

Note: *: $p<0.05$ *vs.* control group; &: $p<0.05$ *vs.* simple DM group; #: $p<0.05$ *vs.* early DN group.

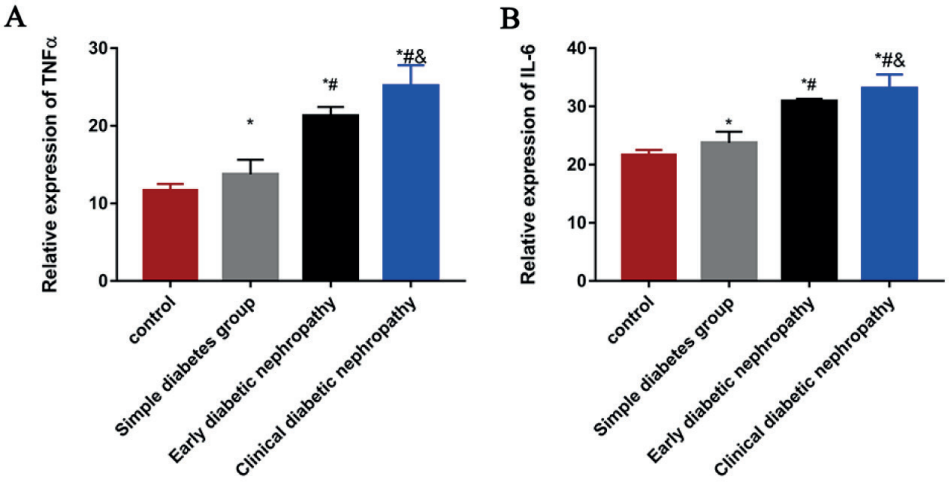

Figure 1. Levels of inflammatory factors (TNF-α and IL-6) in each group of subjects. **A,** Comparisons of TNF-α levels among groups. **B,** Comparisons of IL-6 levels among groups. *: $p<0.05$ *vs.* control group; &: $p<0.05$ *vs.* simple DM group; #: $p<0.05$ *vs.* early DN group.

group than that in the control group (Figure 3), indicating that insulin resistance occurred in the DM and DN group. The ratio of kidney weight to body weight (KW/BW) was evidently higher in the DN group in comparison with that in the control group and DM group (Figure 4).

Discussion

To date, the pathogenesis of DN remains unclear. Increasing evidence has suggested that activation of the complement system is involved in DN. The increased C3, C4, C5, C6, C8 and C9 levels after ischemia are found in the Zucker rat model of T2DM[12]. The elevated serum and renal complement C3 levels are observed in diabetic patients with kidney disease[13,14]. Increased MBL levels have been reported to be associated with an increased risk of DN and a significantly increased risk of death in patients with T1DM. To our knowledge, the role of MASP-2, a key molecule in the lectin pathway, in T2DN is rarely reported. In addition, it is unclear whether complement component expression in the kidney is increased during the process of DN.

Complement molecules are mainly produced by hepatocytes. Zhou et al[15] have shown that extrahepatic tissues including kidney, brain, blood vessels, lungs and intestines can synthesize a small amount of complement components. Among them, the kidney is one of the main sites

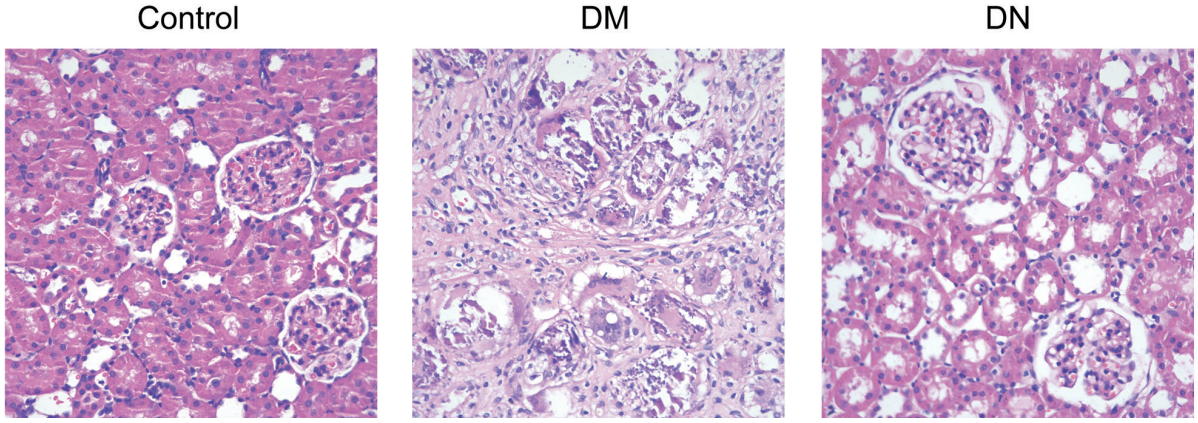

Figure 2. Pathomorphological characteristics. Pathomorphological characteristics of renal arterioles in each group. Arrow indicates thickening of the arteriolar wall (magnification: 200×).

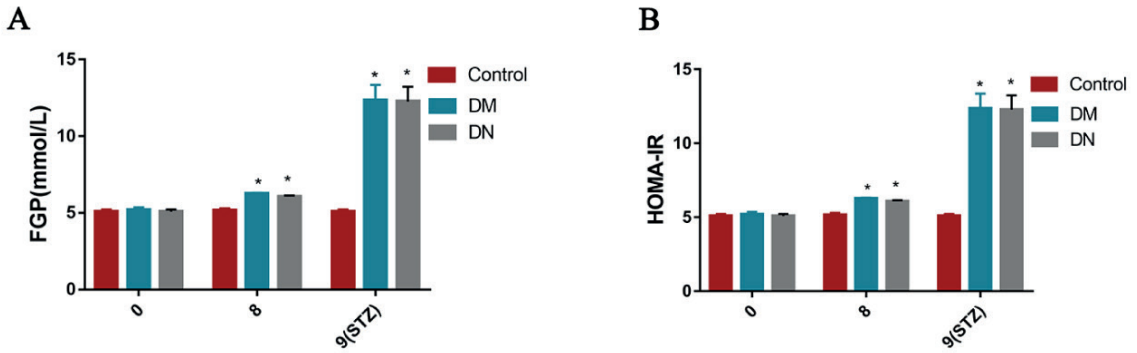

Figure 3. Biochemical indicators during the modeling of T2DN. During the modeling process, FPG (**A**) and insulin resistance index (**B**). *: $p<0.05$ *vs.* control group

for synthesizing complements. Endothelial cells, epithelial cells and renal tubular cells all have the ability to synthesize complement molecules. The expression of complement in the kidney has been implicated in many kidney diseases. Local synthesis of complements (including C1q, C1r, C1s and C3) is increased.

The expressions of complement components in the kidneys of T2DN rats were analyzed in the current study. Most complement components in rat kidney tissues in DN, including C1q, MBL, MASP-2, factor B, C3 and C5b-9, were elevated. In addition, they are correlated with 24 h-UAE, MI and AI levels. The expressions of complement components in the kidney significantly increased in DN, but not in DM, suggesting that the complement system aggravated the process of DN, rather than initiating the process.

Glomerular damage is often considered to be the leading cause of microalbuminuria and early kidney damage[16]. It is reported that immune activity of apoptotic proteins (such as Bax and caspase-3) in glomeruli in the untreated diabetic group is stimulated[17]. Large deposits of lipid peroxidation biomarkers are found in the glomeruli of T2DM rats. The research prompts that tubular injury is the main cause of early kidney diseases. Albuminuria occurs prior to glomerular disease and urinary disease, and albumin is a sensitive indicator of early tubular injury[18]. In proximal tubule cells, increased absorption of renal proteins can generate cytokines and chemokines, which is able to enhance the inflammatory response and activate interstitial fibrosis[19]. The complement system is found to be activated in patients with DN. C1q, C4d and C5b-9 depositions in glomeruli are more common in patients with DN than those without DN, and glomerular C4d and C5b-9 depositions are correlated with severity of DN[20]. Studies[21-23] have indicated that positive expressions of C1q, C3c, C4c, C5, C9 and H factor are found in the renal cortex and medulla of sheep with acute kidney

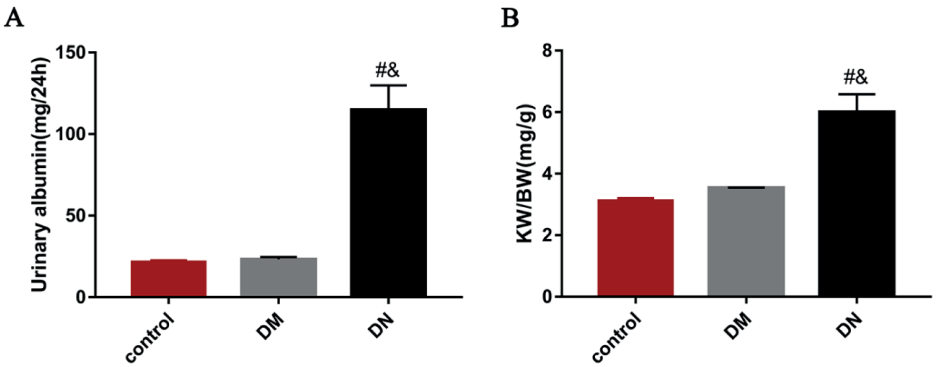

Figure 4. Biochemical indicators during the modeling of T2DN. **A,** 24 h-UAE in each group of rats. **B,** KW/BW in each group. Data are expressed by mean ± **SD. n=8.** #: $p<0.05$ *vs.* control group; &: $p<0.05$ *vs.* DM group.

Table III. Expression level of serum complement C1q in each group of subjects.group of subjects.

Group	Control group	Simple DM group	Early DN group	Clinical DN group
Case	32	42	45	30
C1q	184.02±39.88	189.02±31.88	219.02±41.32	239.02±43.88
t		0.724	183.12	199.21
p		>0.05	<0.05	<0.01

injury. In the current research, most complement components were expressed in the renal tubules, suggesting that the activated complement system was of significance in renal tubular injury than glomerular injury in DN.

By analyzing clinical data in recruited subjects, it is found that Trx and Txnip levels were closely related to the severity of DN. Therein, the clinical DN group had the lowest Trx and the highest level of Txnip. It is suggested that Trx and Txnip could be used to assess the disease progression and prognosis in DN[24].

This study existed some limitations. Due to a small sample size, circulating complement components were not measured. The relationship between the complement system and the progression of DN required to be further validated. In future studies, complement inhibitors, such as C5 inhibitors, anti-MASP-2 monoclonal antibody (AbD04211) or complement-knockout models, will be utilized.

Conclusions

The novelty of this study was that increased expression of complement component in rat kidney is seen in T2DN rats and it is related to the progression of DN. The activated complement system plays a significant role in the progression of T2DN. Therefore, inhibiting the activation of the pathological complement system may be a treatment strategy for DN.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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